EXAMPLE 2

Using the same procedure employed in Example 1, a homogeneous sample of identical bispecific antibody determinants is prepared in which one antibody site is specific for a different antigenic site on the enzyme glucose oxidase from the site for which the bispecific antibody determinant of Example 1 is specific, and in which the second antibody site is specific for an antigenic site on Type I collagen.

EXAMPLE 3

An enzyme electrode for the measurement of lactose is constructed according to the following procedure. First, a collagen membrane shaped to fit over a commercial O_2 electrode is prepared by electrolysis of a collagen fibril suspension using platinum electrodes, as described in Karube et al. (1972) 47, 51–54 BBRC.

A solution is prepared of the bispecific antibody determinants from Example 2 together with a 10-fold or higher molar excess of glucose oxidase, in 0.1 M phosphate buffer, pH 7.0; the glucose oxidase need not be pure. The collagen membrane is immersed in this solution and incubated for 1 h at 20° C., after which time it is rinsed with buffer and then transferred to a solution containing the antibody from Example 1 together with a 10-fold or higher molar excess of β-galactosidase in 0.1 M phosphate buffer, where it is incubated at 20° C. for 1 h. The membrane is then quickly rinsed in buffer and stabilized by immersion in 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.0, for 3 minutes.

The membrane is then placed over the oxygen-permeable teflon membrane of the commercial O_2 electrode, rendering the electrode ready for use for the measurement of lactose, in a manner analogous to the method of measuring sucrose described in Satoh et al. (1976) Biotechnol. and Bioengineering 18, 269–272. A sample containing an unknown amount of lactose is contacted with the membrane, and the immobilized β-galactosidase catalyzes the breakdown of the lactose into glucose, which is then acted on by the immobilized glucose oxidase to release O_2, which is measured as a measure of lactose in the sample.

In the preparation of the membrane described above, molar excesses of enzyme over antibody are employed because β-galactosidase and glucose oxidase are each composed of several identical subunits. An excess of enzyme assures that, on average, only a single antigenic site on each enzyme molecule is involved in complex formation. In the preparation of other electrode using monomeric enzymes, molar excesses of enzymes are not necessary. When equimolar amounts of enzymes and bispecific antibody determinants are used, the reaction can be allowed to proceed in a single stage.

EXAMPLE 4

The following is a description of an example of the type of assay assembly which employs the production of a colored or fluorescent substance, which can be measured colorimetrically, reflectometrically, or fluorometrically, as a measure of an unknown amount of a substance being assayed.

FIG. **5** is a diagrammatic representation of a colorimetric indicator for lactose. Biotin-substituted regenerated cellulose membrane **10** is used as the support for the immobilized enzymes which participate in the series of reactions by which lactose in a sample generates H_2O_2 to produce a colorimetrically measurable result, which is a measure of the amount of lactose in the sample.

The enzymes are immobilized, as shown in FIG. **5**, by being bonded to three different bispecific antibody determinants, prepared according to the procedure described in Example 1. The first determinant has one site, A′, specific for an antigenic site on the protein avidin, and the other site, B′, specific for an antigenic site on the enzyme horseradish peroxidase. The second determinant has a site, C′, specific for a different antigenic site on horseradish peroxidase, and the second site, D′, specific for an antigenic site on glucose oxidose. The third determinant has an antibody site E′, specific for a different antigenic site on glucose oxidase, and the second site, F′, specific for an antigenic site on β-galactosidase.

Substituted cellulose membrane **10** is prepared by the cyanogen bromide procedure, e.g. Cuatrecasas et al. (1968) Proc. Nat'l. Acad. Sci. U.S.A. 61, 636–643, as follows. Regenerated cellulose membranes are suspended in 0.1 M $NaHCO_3$ at 4° C. and treated with an equal volume of 2.5% CNBr solution, the pH being continuously adjusted to 11 with 2 N NaOH and the temperature kept at 4° C. After 8 min, the cellulose membranes are washed with 0.1 M $NaHCO_3$ and then with water, 50% acetone, and finally with 100% acetone. The cellulose membranes are then incubated at 4° C. for 20 h in 0.2 M $NaHCO_3$, pH 9, containing 1 mg per ml of ε-N-biotinyl-L-lysine (Bayer et al. (1974) *Methods in Enzymology* 34B, 265–267), followed by extensive washing with water.

The biotin-substituted cellulose membrane is then immersed in 0.1 M phosphate buffer, pH 7.0, and incubated for 1 h at 20° C. with approximately equivalent molar amounts of avidin, horseradish peroxidase, and the bispecific antibody determinant having sites A′ and B′. The membrane is then rinsed with buffer and transferred to a solution containing an approximately equivalent molar amount of the bispecific antibody determinant having sites C′ and D′, and a 10-fold molar excess of glucose oxidase. After 1 hour at 20° C., the membrane is rinsed with buffer and transferred to a solution containing an approximately equivalent molar amount of the bispecific antibody determinant having sites E′ and F′, and a 10-fold molar excess of β-galactosidase, and incubated at 20° C. for 1 h, followed by rinsing with buffer. If repeated use is anticipated, the membrane is stabilized by immersion in 0.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7, for 3 min.

The enzymes used in the above-described procedure need not be pure. In the example described, a molar excess of β-galactosidase and glucose oxidase was necessary because these enzymes are composed of several identical subunits. In cases where only monomeric enzymes are used, molar excesses of enzymes are not necessary. When equimolar amounts of enzymes and bispecific antibody determinants are used, the reaction can be allowed to proceed in a single stage.

For the determination of lactose, membrane **10** is immersed in or wetted with a sample containing an unknown amount of lactose in 0.1 M phosphate buffer, pH 7, and 0.01% o-dianisidine.

As shown in FIG. **5**, lactose in the sample first acts on β-galactosidose to form glucose, which in turn is acted on by glucose oxidase, in the presence of oxygen, to release H_2O_2, which, with peroxidase, oxidizes o-dianisidine to produce a yellow dye with absorbance at 460 NM. Various other chromogenic or fluorogenic substances can be substituted for o-dianisidine.